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TITLE OF THE INVENTION (500 characters max)

The Structural Requirements at the Catalytic Site of the Heteroduplex Substrate for Human RNase H1 Catalysis

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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☐ Application Date Sheet. See 37 CFR 1.76

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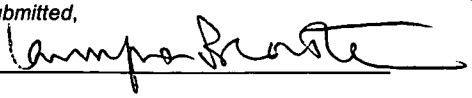
Respectfully submitted,

SIGNATURE 

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Number 2 of 2

The structural requirements at the catalytic site of the heteroduplex substrate for human RNase H1 catalysis

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Introduction

RNase H hydrolyzes RNA in RNA-DNA hybrids (1). RNase H activity appears to be ubiquitous in eukaryotes and bacteria (2-7). Although RNases H constitute a family of proteins of varying molecular weight, the nucleolytic activity and substrate requirements appear to be similar for the various isotypes. For example, all RNases H studied to date function as endonucleases exhibiting limited sequence specificity and requiring divalent cations (e.g., Mg^{2+}, Mn^{2+}) to produce cleavage products with 5→phosphate and 3→ hydroxyl termini (8).

Recently, two human RNase H genes have been cloned and expressed (9-11). RNase H1 is a 286 amino acid protein and is expressed ubiquitously in human cells and tissues (9). The amino acid sequence of human RNase H1 displays strong homology with RNase H1 from yeast, chicken, *E.coli* and mouse (9). The human RNase H2 enzyme is a 299 amino acid protein with a calculated mass of 33.4 kDa and also shown to be ubiquitously expressed in human cells and tissues (10, H. Wu, unpublished data). Human RNase H2 shares strong amino acid sequence homology with RNase H2 from C. elegans, yeast and E. coli (10). Although the biological role for the human enzyme are not fully understood, RNase H2 appears to be involved in *de novo* DNA replication and RNase H1 has been shown in mice to be important for mitochondrial DNA replication (12).

Human RNase H1 has been shown to play a dominant role in the activity of DNA-like antisense oligonucleotides (Wu et al., in press). Human RNase H1 and H2 proteins were both overexpressed in several cell lines and mouse liver and the levels of each enzyme

reduced by using DNA-like antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) targeting each of the enzymes. The effects of these manipulations on the potencies of a number of DNA-like ASOs to several different target RNAs showed that increasing the level and activity of human RNase H1 increased the potency of the ASOs, while increasing the level and activity of RNase H2 had no effect on the IC_{50} values for several DNA-like ASOs to different targets (Wu et al, in press, Wu unpublished). Moreover, overexpression of human RNase H1 in mouse liver but not RNase H2 increased the potency of a DNA-like ASO targeting Fas after IV administration. Further, reducing the level and activity of RNase H1 reduced the potencies of the ASOs, while reducing RNase H2 had no effect (Wu et al, in press, Wu unpublished).

The structure of human RNase H1 was shown to consist of a 73 amino acid region homologous with the RNA-binding domain of yeast RNase H1 at the amino-terminus of the protein and separated from the catalytic domain by a 62 amino acid spacer region (13-15). The catalytic domain is highly conserved with the amino acid sequences of other RNase H1 proteins and contains the key catalytic and substrate binding residues required for activity (13, 16-19). Site-directed mutagenesis of human RNase H1 revealed that the spacer region was required for RNase H activity (13). Although the RNA-binding domain was shown not to be required for RNase H activity, this region was responsible for the enhanced binding affinity of the human enzyme for the heteroduplex substrate as well as the strong positional preference for cleavage exhibited by the enzyme (13, 20). The RNA-binding domain of human RNase H1 is conserved in other eukaryotic RNases H1 and the highly conserved lysines at positions 59 and 60 of human RNase H1 have been

shown to be important for binding to the heteroduplex substrate (20). The conserved tryptophan at position 43 was responsible for properly positioning the enzyme on the substrate for catalysis (20).

Human RNase H1 exhibits a strong positional preference for cleavage, i.e., human RNase H1 cleaves the heteroduplex substrate between 7 to 12 nucleotides from the 5'-RNA/3'-DNA terminus (13 and 20). Based on site-directed mutagenesis of both human RNase H1 and the heteroduplex substrate the RNA-binding domain was shown to be responsible for the observed positional preference for cleavage. The RNA-binding domain of human RNase H1 appeared to bind to the at the 3'-DNA/5'-RNA pole of the heteroduplex substrate with the catalytic site of the enzyme positioned slightly less than one helical turn from the RNA-binding domain (20). Substitution of either the terminal 3'-DNA with a single ribonucleotide or 5'-RNA with a 2'-methoxyethoxy deoxyribonucleotide was shown to cause a concomitant 3'-shift of the first 5'-cleavage site on the RNA, suggesting that altering duplex geometry interferes with proper positioning of the enzyme on the heteroduplex for cleavage (20). Although the interaction between the RNA-binding domain and the heteroduplex substrate has been characterized, the mechanism by which the catalytic domain of RNase H1 recognizes the substrate is unclear.

Human RNase H1 is a nuclease that cleaves RNA exclusively in an RNA/DNA duplex via a double-strand DNase cleavage mechanism. Neither double-strand RNA (dsRNA) or DNA (dsDNA) duplexes support RNase H1 activity (21-22). The observed structural differences between the RNA/DNA heteroduplex and dsRNA and dsDNA duplexes

suggest a possible role for the helical geometry and the sugar conformation of the DNA and RNA in the selective recognition of the heteroduplex substrate by human RNase H1 (23-25). Specifically, the deoxyribonucleotides within dsDNA form a southern C2-*endo* sugar conformation resulting in a B-form helical conformation, whereas ribonucleotides within dsRNA form a northern C3-*endo* pucker and an A-form helical geometry. In contrast, the deoxyribonucleotides of the RNA/DNA heteroduplex have been shown to adopt an eastern O4-*endo* sugar pucker resulting in a helical conformation where the RNA strand adopts A-form geometry and the DNA strand shares both the A- and B-form helical conformations. The conformational diversity observed for the DNA strand is likely a function of the intrinsic flexibility of the deoxyribonucleotide compared to RNA, and may also be important for human RNase H1 activity. DNA also differs from RNA in that the furanose ring of deoxynucleotides are much more flexible, i.e., exhibit a nearly symmetrical potential energy barrier for both south and north sugar conformations(25).

Consistent with these observations, heteroduplexes containing 2'-ara-fluoro deoxyribonucleotides, which have been shown to exhibit a sugar conformation comparable to DNA when hybridized to RNA, have also been shown to support RNase H1 activity (26). On the other hand, heteroduplexes consisting of RNA/2'-alkoxy modified deoxyribonucleotides, exhibiting C3-*endo* sugar pucker and an A-form helical geometry when hybridized to RNA do not support human RNase H1 activity (21-22). We have previously shown that both *E. coli* and human RNases H1 bind A-form duplexes (e.g., RNA/RNA, 2'-methoxyethoxy/RNA and 2'-methoxy/RNA) with comparable affinity to the DNA/RNA heteroduplex substrate but did not cleave the A-form duplexes

(21-22). In this case, the size and position of the 2'-substituents of RNA and 2'-alkoxy nucleotides suggest possible steric interference with RNase H1 as the 2'-substituents are positioned within the minor groove of the heteroduplex; a region predicted to be the binding site for the enzyme (27).

In the present study we determined the structure activity relationships for the interaction between the catalytic domain of human RNase H1 and the RNA/DNA heteroduplex substrate by systematically evaluating the influence of sugar conformation, bulk in the minor groove and flexibility in the catalytic area on enzyme efficiency. Modified nucleotides were introduced into the oligodeoxyribonucleotides at the human RNase H1 preferred cleavage sites on the heteroduplex and consisted of the DNA-like southern C2-*endo*, RNA-like northern C3-*endo* and eastern O4-*endo* biased sugars with and without 2'-substituents (Fig. 1A). In addition, varying degrees of conformational flexibility were introduced into the heteroduplex substrate by incorporating modified deoxyribonucleotides that →stack with the adjacent deoxyribonucleotides but do not form hydrogen bonds with the bases of the RNA strand, abasic deoxynucleotides, hydrocarbon intranucleotide linkers and the gancycloviar modified deoxyribonucleotide (Fig. 1B). The initial cleavage rates (V_0) as well as site-specific cleavage rates for the modified heteroduplexes were compared with the wild type DNA/RNA heteroduplex.

Materials and Methods

Preparation of Human RNase H1.

Human RNase H1 was expressed and purified as previously described (28). Briefly, the plasmids were transfected into *E. coli* BL21 (DE3) (Novagen, WI). The bacteria was grown in Terrific Broth medium (Boi 101 Systems) at 37° C and harzested at OD_{600} of 1.2. The cells were induced with 1 mM isoprpylthiogalactoside (IPTG) at 37° C for 2 h. The cells were lysed in 6 M guanidine hydrochoride 100m mM sodium phosphate, 10 mM tris, pH 8.0 for 16 – 20 h at 24° C. The recombinant proteins were incubated for 1 h with 1 mL of Ni-NTA Super flow beads (Qiagen) per 50 mL of lysate. The Ni-NTA media was packed into an FPLC column and the RNase H1 proteins partially purified with sequential gradients (flow rate, 5 mL/min; buffer A, 100 mM sodium phosphate, 10 mM tris-HCL, 8 M Urea, pH 6.3; buffer B, 100 mM sodium phosphate, 10 mM tris-HCL, 2 M Urea, pH 6.3; buffer C, 100 mM sodium phosphate, 10 mM tris-HCL, 2 M Urea, 100 mM EDTA, pH 7.0). The eluent was further purified by ion exchange FPLC chromatography (Mono S Column; flow rate, 1 mL/min; buffer A, 20 mM sodium phosphate, 2 M urea, 200 mM NaCl, pH 7.0; buffer B, 20 mM sodium phosphate, 2 M urea, 2 M NaCl, pH 7.0). Fractions containing RNase H1 were pooled and concentrated. The concentrated protein was purified by RP-FPLC (Resourse RPC Column; flow rate 1 mL/min; Buffer A, 2% acetonirtile in diH_2O, 0.065% triflouro acid; buffer B 80% acetonitrile in diH_2O, 0.05% trifluoro acid). Fractions were lyophilized, resuspended in diH_2O and analyzed by SDS-PAGE.

Synthesis of oligonucleotides

The oligoribonucleotides were synthesized on a PE-ABI 380B synthesizer using 5'-*O*-silyl-2'-*O*-bis(2-acetoxyethoxy)methyl ribonucleoside phosphoramidites and procedures

described elsewhere (29). The oligoribonucleotides were purified by reverse-phase HPLC. The oligodeoxyribonucleotides were synthesized on a PE-ABI 380B automated DNA synthesizer and standard phosphoramidite chemistry. The oligodeoxyribonucleotides were purified by precipitation 2 times out of 0.5 M NaCl with 2.5 volumes of ethyl alcohol. The 3'-modified oligonuleotides were synthesized with CPG (Glen Research, Sterling, VA) containing either the 3'-deoxy or 2',3'-dideoxy nucleotide.

The 1,4-anhydro-5-*O*-(4,4'-dimethoxytrityl)-2-deoxy-D-erythro-pentitol -3-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite], 5'-*O*-(4,4'-dimethoxytrityl)]-3-(4-methylbenzoyl)-2-thio-thymidine-3'-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite, 5'-*O*-(4,4'-dimethoxytrityl)-3'-deoxypseuodouridine-3'-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite, 1',2'-dideoxy-1'-(2,4-difluorotolyl)-5'-*O*-(4,4'-dimethoxytrityl)-→D-ribofuranose-3'-[(2-cyanoethyl)-*N,N*-diisopropyl]pho sphoramidite and 5'-*O*-(4,4'-dimethoxytrityl)]-3-(4-methylbenzoyl)-2'-uridine-3'-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite were procured form commercial sources (Glen Reseaech Inc., Viriginia, U. S. A). The 1-[2-deoxy-2-fluoro-5-*O*-(4,4'-dimethoxytrityl)]-3-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite-→D-[arabinofur anosyl]-thymine, 5'-*O*-(4,4'-dimethoxytrityl)]-2'-deoxy-2'-fluoro-thymidine-3'-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite and 5'-*O*-(4,4'-dimethoxytrityl)]-2'-S-methyl-2'-thio-5-methyluridine-3'-[(2-cyanoethyl)-*N,N*-diisopropyl]phosphoramidite were synthesized as reported (30-33). Standard phosphoramidites and solid supports were used for incorporation of A, T, G, and C residues. A 0.1 M solution of the amidites in anhydrous

acetonitrile was used for the synthesis of modified oligonucleotides. The oligonucleotides were synthesized on functionalized controlled pore glass (CPG) on an automated solid phase DNA synthesizer with final DMT group retained at 5'-end. For incorporation of modified amidites, 6 equivalent of phosphoramidite solutions were delivered in two portions, each followed by a 3 min coupling wait time. All other steps in the protocol supplied by the manufacturer were used without modification. Oxidation of the internucleotide phosphite to the phosphate was carried out using *tert*-butyl hydroperoxide/acetonitrile/water (10 : 87 : 3) with 10 min oxidation wait time. The coupling efficiencies were more than 97%. To deprotect oligonucleotides containing 2'-deoxy-2'-fluoro-thymidine and 2-deoxy-2-fluoroarabinofuranosylthymine, the solid support bearing the oligonucleotides were suspended in aqueous ammonia (28-30 wt%):ethanol (3:1, 3 mL for 2 μmol scale synthesis) and heated at 55 °C for 6 h. All other modified oligonucleotides after completion of the synthesis, the solid supports bearing the oligonucleotides were suspended in aqueous ammonium hydroxide (28-30 wt %, 2 mL for 2 μmol scale synthesis) and kept at room temperature for 2 h. The solid support was filtered and the filtrate was heated at 55 °C for 6 h to complete the removal of all protecting groups. Crude oligonucleotides were purified by high performance liquid chromatography (HPLC, C-4 column, Waters, 7.8 x 300 mm, A = 100 mM ammonium acetate, pH 6.5-7, B = acetonitrile, 5-60% of B in 55 min, flow 2.5 mL min^{-1}, →260 nm). Detritylation was achieved by adjusting the pH of the solution to 3.8 with acetic acid and keeping at room temperature until complete removal of the trityl group, as monitored by HPLC analysis. The oligonucleotides were then desalted by HPLC to yield modified oligonucleotides in 30-40% isolated yield calculated based on the loading of the 3'-base

to solid suppor (34). The oligonucleotides were characterized by electrospray mass spectroscopy (ES-MS) and their purity was assessed by HPLC and capillary gel electrophoresis (CGE). The purity of the oligonucleotides was > 90%.

The nucleoside 3'-→*C*-methylthymidine was synthesized from 1,2-*O*-isopropylidene-D-xylofuranose and converted to the 5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-(2-cyanoethyl-diisopropylamino)-phosphoramidite as previously described (35). This phosphoramidite was incorporated into oligonucleotides using an ABI 394 synthesizer with standard reagents and conditions, except for the use of a double-coupling procedure (two pulsed deliveries of amidite during a 10 minute coupling step). The 4'-→C-methylthymidine nucleoside was synthesized in 12 steps (manuscript in preparation) starting from commercially available 1,2:5,6-Di-O-isopropylidene-→D-glucofuranose purchased from Pfanstiehl, Waukegan, IL. The alternate synthesis of this nucleoside has been reported (36-37). It was converted to 5'-*O*-(4,4'-dimethoxytrityl)-4'-→C-methylthymidine-3'-*O*-(2-cyanoethyl)-N, N-Diisopropylphosphoramidite by following the procedure described for similar compounds (38-39). The phosphoramidite of this modification was incorporated into oligonucleotide by the same method described above.

The oligodeoxyribonucleotides containing the 2'-ara-fluoro, gancyclovir, abasic deoxyribonucleotide, 2-fluoro-6-methylbenimidazole deoxyribonucleotide, 4-methylbenimidazole deoxyribonucleotide and hydrocarbon linkers were synthesized in 3 μmole scale on ABI synthesizer (Model 398B), using standard phosphoramidite method and 0.2M solutions of the corresponding 3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-

phosphoramidite. Oligomerization of nonstandard phosphoroamidites was performed using double coupling (25 equivalents per coupling) and 10 minutes coupling time. Phosphorodiester linkages were introduced by oxidation with a 0.1 M solution of iodine in THF/Pyridine/water (80/20/1, v/v/v). The 2'-Deoxy-2'-fluoro-→D-arabinonucleoside containing oligonucleotides were deprotected using two step procedure involving: deprotection in concentrated NH_4OH (20min., at 55°C), followed by 2 hrs treatment with mixture of methyl amine/NH4OH (1/3, v/v, 2hrs at 55°C). All of other modified and unmodified, oligonucleotides were cleaved from the support and deprotected by treatment with concentrated NH_4OH at 55°C for 16hrs, and evaporated to dryness. Oligonucleotide products were resuspended in water and purified and desalted by RP-HPLC, using DELTA PACK column (Waters, 300x7.8 mm I.D., C18 μm, 300A. Structural identity and homogenity was confirmed by CE, RP-HPLC and ES-MS.

Oligonucleotides with abasic sites were conveniently generated by the use of uracil-DNA glycosylase (40). Oligonucleotides containing uridine residue were synthesized as described (vide supra). The HPLC purified oligonucleotides (0.32 mg) were dissolved in Uracil-DNA Glycosylase (149 μl, I unit in 1 μl dissolved in 30 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% Tween 20 and 50% glycerol) and incubated at 37 °C for 4 h. The reaction was terminated by filtering the enzyme using low binding membrane filter (0.22 μm, Millipore Inc., Bedford, MA, USA). The release of uracil was observed in HPLC analysis of the reaction mixture (Waters C-4 3.9 x 300 mm, , delta pack, 15 micron, 300 A°, A = 100mM ammonium acetate, B = acetonitrile 0 to 25 % B in 55 min, flow 1.5 ml min^{-1}, →260 nm) and co injecting the authentic sample.

The oligonucleotides were purified by HPLC (conditions same above). The purity (> 90%) of the oligonucleotides was assed by HPLC analysis.

Preparation of ^{32}P labeled substrate

The RNA substrate was 5'-end-labeled with ^{32}P using 20 u of T4 polynucleotide kinase (Promega, WI), 120 pmol (7000 Ci/mmol) [γ-^{32}P]ATP (ICN, CA), 40 pmol RNA, 70 mM tris, pH 7.6, 10 mM $MgCl_2$ and 50 mM DTT. The kinase reaction was incubated at 37° C for 30 min. The labeled oligoribonucleotide was purified by electrophoresis on a 12% denaturing polyacrylamide gel (41). The specific activity of the labeled oligonucleotide is approximately 3000 to 8000 cpm/fmol.

Preparation of the heteroduplex

The heteroduplex substrate was prepared in 100 μL containing unlabeled oligoribonucleotide ranging from 100 to 1000 nM, 10^5 cpm of ^{32}P labeled oligoribonucleotide, two-fold excess complementary oligodeoxyribonucleotide and hybridization buffer [20 mM tris, pH 7.5, 20 mM KCl]. Reactions were heated at 90° C for 5 min, cooled to 37° C and 60 u of Prime RNase Inhibitor (5 Prime → 3 Prime, CO) and $MgCl_2$ at a final concentration of 1mM were added. Hybridization reactions were incubated 2 - 16 h at 37° C and 1 mM tris(2-carboxyethyl)phosphate (TCEP) was added.

Multiple-turnover kinetics

The human RNase H1 proteins were incubated with dilution buffer (50 mM tris, 50 mM NaCl, 100 μM TCEP, pH 7.5) for 1 h at 24° C. The heteroduplex substrate was digested

with 0.4 ng of enzyme at 37° C. A 10 μl aliquot of the cleavage reaction was removed at time points ranging from 2 - 120 min and quenched by adding 5 μL of stop solution (8 M urea and 120 mM EDTA). The aliquots were heated at 90° C for 2 min, resolved in a 12% denaturing polyacrylamide gel and the substrate and product bands were quantitated on a Molecular Dynamics PhosphorImager. The concentration of the converted product was plotted as a function of time. The initial cleavage rate (V_0) was obtained from the slope (mole RNA cleaved/min) of the best-fit line for the linear portion of the plot, which comprises, in general $< 10\%$ of the total reaction and data from at least five time points. Site-specific cleavage rates were determined by plotting the concentration of the converted product for a given cleavage site as a function of time.

Results

Oligodeoxyribonucleotides were prepared with nucleotide modifications positioned within the region preferentially cleaved by human RNase H1 (Fig. 1C). The deoxyribonucleotide modifications consisted of sugar modifications (e.g., 2'-methylthiothymidine, 2'-ara-fluoropyrimidine, 4'-methylthymidine, 3'-methylthymidine, cyclohexenylthymidine and 2'-fluorothymidine) as well as base modifications (e.g., 2-thiothymidine, pseudo-uridine, 2-fluoro-6-methylbenimidazole deoxyribonucleotide, 4-methylbenimidazole deoxyribonucleotide and 2,4-difluorotolyl deoxyribonucleotide) (Fig, 1). In addition, the deoxyribonucleotides were substituted with hydrocarbon linkers ranging in length from 3 to 5 residues, gancyclovair, tetrahydrofuran and abasic deoxyribonucleotide and (Fig. 1B). The modified oligodeoxyribonucleotides were

annealed to complementary RNA and the heteroduplexes digested with human RNase H1 under multiple-turnover conditions as described in materials and methods. Initial cleavage rates (V_0) as well as site-specific cleavage rates, i.e., initial cleavage rates for each human RNase H1 cleavage site, were determined (Table I).

Figure 2 shows examples of the human RNase H1 cleavage patterns and site-specific cleavage rates for the unmodified substrate and heteroduplexes containing the 4'-methylthymidine at position T_9 and 2-thiothymidine at position T_8. The 2-thiothymidine modificed deoxyribonucleotide ablated of the site-specific cleavage rates for the ribonucleotide apposing the modified nucleotide and the adjacent 3'-ribonucleotide, i.e., positions 0 and +1, resulting in a 30 percent reduction in the V_0 observed for this heteroduplex (Fig. 2 and Table 1A). The 4'-methylthymidine modification exhibited a significantly greater effect on human RNase H1 activity. In this case, no human RNase H1 cleavage was also observed for the ribonucleotide apposing the 4'-methylthymidine and the three adjacent 3' and two adjacent 5'-ribonucleotides, i.e., positions -2 to +3 (Fig. 2 and Table 1A). In addition, all the site-specific cleavage rates for the heteroduplex containing the 4'-methylthymidine were slower when compared to the unmodified substrate, resulting in a 3-fold reduction in the initial cleavage rate (Table I). Together these data suggest that the degrees to which the initial cleavage rates were reduced for the modified heteroduplexes was a function of the number of human RNase H1 cleavage sites either reduced or ablated (Table I).

The initial cleavage rates (V_0) observed for the modified heteroduplexes were predominantly dependant on the class of modification tested rather the position of the specific modification within the oligodeoxynucleotide (Table I). For example, several modified heteroduplexes (e.g., 4'-methylthymidine, cyclohexenylthymidine, tetrahydrafuran, gancyclovir and the hydrocarbon linkers) exhibited initial cleavage rates 2 to 3-fold slower than the V_0 of the unmodified substrate whereas the 2'-ara-flouropyrimidine, pseudouridine and →stacking deoxyribonucleotides, (e.g., 2-fluoro-6-methylbenimidazole, 4-methylbenimidazole and 2,4-difluorotolyl deoxyribonucleotides) modified heteroduplexes exhibited initial cleavage rates comparable to the rate observed for the unmodified substrate (Table I). It is important to note that a 2-fold reduction of the initial cleavage rate due to a single nucleotide modification is significant considering that human RNase H1 cleaves the substrate at multiple positions within the heteroduplex. In contrast, the initial cleavage rates for the heteroduplexes containing the same modification at different positions within the substrate showed only a ±10 percent difference in the cleavage rates (Table I).

The modifications that exhibited the greatest impact on the site-specific cleavage rate for the ribonucleotide apposing the modification also exhibited the broadest effect on the site-specific cleavage rates surrounding ribonucleotides. The tetrahydrofuran, hydrocarbon linkers, 4'-methylthymidine and abasic deoxyribonucleotide modifications which exhibited significantly reduced or ablated the site-specific cleavage rates for the ribonucleotide apposing the modification also showed significantly slower site-specific cleavage rates for the surrounding 3' and 5'-ribonucleotides, (e.g., positions –2 to +2)

compared with the unmodified substrate (Table I). Interestingly, with the exception of 4'-methylthymidine, these modifications were predicted to impart the greatest degree of conformational flexibility at the site of the modification. The heteroduplexes containing the pseudouridine, 2'-ara-fluoropyrimidine, 3'-methylthymidine, cyclohexenylthymidine and →stacking deoxyribonucleotides modifications which exhibited little to no reduction in the site-specific cleavage rate for the ribonucleotide apposing the modification also showed only a modest reduction in the site-specific rates for the surrounding ribonucleotides (Table I). Interestingly, for a majority the modified deoxyribonucleotides tested, the influence on the human RNase H1 activity of the adjacent ribonucleotides appeared to be unidirectional (Fig. 1A). For example, the cyclohexenylthymidine, 2'-methylthiothymidine, 3'-methylthymidine, 2-thiothymidine, 2'-fluorothymidine and pseudouridine reduced the site-specific cleavage rates for the adjacent 3'-ribonucleotides more significantly than the 5'-ribonucleotides.

Discussion

Within the context of nucleic acid duplexes, the pseudorotation of the sugar from the southern to the northern conformation sets into motion a series of structural changes that ultimately result in the formation of B-form or A-form duplexes. The shift in helical conformation resulting from the pseudorotation of the sugar starts with the change in the torsion angles of the glycosyl and C4-C5 bonds. The spatial orientation of the of the glycosyl bond in turn dictates the distance between the internucleotide phosphates, the rotation and axial rise per nucleotide, the tilt of the base-pair and the dislocation of the

base pairs from the helical axis. These factors combined, control the depth and width of the major and minor grooves (for review see: 25).

RNA/DNA heteroduplexes are unique in that these duplexes exhibit an intermediate structure between the canonical A-form and B-form helical geometry. At approximately 9Å the minor groove width for the heteroduplex is approximately midway between the minor groove widths for A-form (∏11Å) and B-form (∏6Å) duplexes (23-25). The RNA sugars of the heteroduplex exhibit a northern sugar conformation whereas the DNA sugars form an eastern O4'-*endo* sugar conformation. The intrastrand phosphate distances for the heteroduplex also differ from canonical A-form and B-form duplexes in that the DNA strand maintains a intrastrand phosphate distance consistent with B-form duplexes whereas the intrastrand phosphate distance within the RNA strand is closer to an A-form duplex. Finally, the unique minor groove width as well as the position of the inter- and intrastrand phosphates exhibited by the DNA/RNA heteroduplexes suggest that these features may be the key recognition determinants for RNase H enzymes.

Although, the key catalytic amino acids of human RNase H1 have been identified, the structural and physical properties of the enzyme and substrate responsible for the selective recognition and cleavage of the RNA in the RNA/DNA heteroduplex are not known as a cocrystal structure of the enzyme/substrate complex has not been solved (13). Site-directed mutagenesis of the *E. coli* and human RNase H1 enzymes combined with molecular modeling of the enzyme/substrate complex suggest that the enzyme binds to the minor groove of the heteroduplex substrate (42, 13, Lima unpublished). In addition,

the catalytic site of the enzyme was predicted to contact the 2'-hydroxyls of the RNA strand and the phosphates of the DNA strand surrounding the scissile phosphate (Fig. 3).

In this study we performed a complementary mutational analysis on the structure the substrate at the catalytic site for human RNase H1. We designed a series of modified heteroduplexes with the modifications positioned within the catalytic site of the substrate (Fig. 1). The modifications consisted of nucleotides exhibiting northern, southern and eastern sugar conformations, base modifications which →stack with adjacent nucleotides but do not form hydrogen bonds, abasic deoxynucleotides, internucleotide hydrocarbon linkers ranging 3-5 residues and gancycloviar substitution of the deoxyribose to determine the role of helical geometry, sugar conformation, bulk in the minor groove and conformational flexibility within the heteroduplex on human RNase H1 activity.

The role of sugar conformation within the catalytic site of the heteroduplex

The northern biased deoxyribonucleotides selected for this study included the 2'-fluorothymidine, 2-thiotymidine and cyclohexenylthymidine modifications (Fig. 1A). All three modifications lacked bulky 2'-substituents in order to avoid possible steric interactions with the enzyme. These modifications were determined to influence sugar conformation through distinctly different mechanisms. For example, the highly electronegative fluorine of the 2'-fluorothymidine acts in conjunction with the gauche effect to strongly stabilize the sugar in the northern pucker (30). In the case of 2-thiothymidine, it has been shown at the dinucleotide level that the highly polorizable

sulfer stabilizes the C3'-*endo* sugar conformation as well as the stacking interactions with the neighboring nucleotides and imparts stronger hydrogen bonding due to the increased acidity of the N-3 imino proton (43-45). The cyclohexenyl nucleic acids differ from the other northern biased deoxyribonucleotides in that the furanose moiety is replaced with a hexene ring. The hexene ring offers an attractive alternative to furanose as the flexibility of the hexene is similar to the furanose ring and can adopt two extreme half-chair conformations $^3H_2$ and $^2H_3$ that mimic, respectively, the C3'-*endo* and C2'-*endo* puckered furanose (46). When incorporated into a oligodeoxyribonucleotide and hybridized to RNA, the hexene ring has been shown to adopt the $^3H_2$ northern-like ring conformation (46). All three modifications exhibited similar effects on the site-specific cleavage rates. The heteroduplexes containing the northern biased modifications showed significantly slower site-specific cleavage rates for the ribonucleotide apposing the modification (position 0) (Table IA). Further, these modified heteroduplexes also exhibited significantly slower site-specific cleavage rates for the adjacent 3'-ribonucleotide, i.e., position +1, as little to no reduction for the site-specific cleavage rates was observed for the adjacent 5'-ribonucleotide, i.e., position –1, these modifications appear to be influenecing the structure of the adjacent base-pairs in a unidirectional manner (Table IA). Thus, these results demonstrate that the conformation of the heteroduplex is a significant determinant of the suitability of a substrate independent of minor groove bulk.

The role of the minor groove substituents

In contrast to northern biased deoxyribonucleotides, modified deoxyribonucleotides exhibiting a southern biased sugar pucker more closely mimic the sugar conformation of native deoxyribonucleosides. Surprisingly, the effects of southern biased deoxyribonucleotide modifications on RNase H1 activity have not been previously investigated. We evaluated the human RNase H1 activity for heteroduplexes containing the southern biased deoxyribonucleotide modifications. Heteroduplexes containing the 2'-methylthiothymidine modifications were poor substrates for human RNase H1 exhibiting significantly slower initial cleavage rates (V_0) and site-specific cleavage rates for both the apposing and adjacent ribonucleotides (Table IA). The 2'-methylthiothymidine nucleoside are highly southern biased as a result of the electronegativity and steric bulk of the 2'-substituent. The 2'methylthio substituent potentially poses a similar steric problem for the enzyme as the 2'-alkoxy moieties and may account for the observed loss in human RNase H1 activity. These data suggest that, consistent with the predicted binding model, human RNase H1 is likely interacting with the minor groove of the heteroduplex substrate.

The role of the deoxyribonucleotide phosphate groups

Heteroduplexes containing the 4'-methylthymidine modifications were also poor substrates for human RNase H1 exhibiting initial cleavage rates 2 to 3-fold slower than the unmodified heteroduplex (Table IA). The 4'-methylthymidine inhibited the human RNase H1 cleavage of the ribonucleotide apposing the modification and the adjacent 3'-ribonucleotides. The site-specific rates for the adjacent 5'-ribonucleotides were

significantly reduced compared to the unmodified substrate. In contrast, the heteroduplexes containing the 3'-methyl modified deoxyribonucleotide, which have been shown to exhibit a sugar conformation similar to the 4'-methyl nucleosides, were significantly better substrates for human RNase H1. The observed differences in the human RNase H1 activity for the 4'-methylthymidine and 3'-methylthymidine heteroduplexes may be a function of the position of the 4'-methyl moiety on the furansose ring, which is predicted to sterically alter the position of the phosphate group on the modified deoxyribonucleotide (ref). Furthermore, the loss in human RNase H1 activity observed for the 4'-methylthymidine heteroduplexes suggests that proper orientation of the phosphate group on the deoxyribonucleotide apposing the scissile ribonucleotide is important for human RNase H1 activity. Finally, the results from 3'-methylthymidine modified heteroduplexes suggest that heteroduplexes containing southern biased sugars in the DNA strand are better tolerated by human RNase H1 than northern biased deoxyribonucleotides.

Optimal modification mimic the conformation of deoxyribonucleotides

The nucleotide modifications predicted to mimic the sugar pucker of the deoxyribonucleotide of an RNA/DNA heteroduplex, (e.g., heteroduplexes containing the 2'-ara-fluoropyrimidines and pseudouridine modifications) exhibited cleavage rates comparable to the rates observed for the unmodified substrate (Table IA). The 2'-ara-fluoro modification has been shown by NMR to form the eastern O4'-*endo* sugar conformation similar to DNA when hybridized to RNA (26). In addition, the size and

position of the 2'-ara-substituent, i.e., the fluorine is directed upward and away from the minor groove, is predicted not to sterically interfere with the enzyme. In the case of the pseudouridine, the NMR structure indicated a modestly higher southern sugar pucker population for the nucleoside due to the influence of the torsion angle of the C-C glycosyl bond on the dyhydryl angle between 1' and 2'-carbons (47). Furthermore, the CD spectrum for RNA/pseudouridine and DNA/pseudouridine duplexes showed, respectively, an RNA-like C3-*endo* and DNA-like C2-*endo* sugar pucker for the pseudouridine deoxyribonucleotides suggesting that pseudouridine exhibits a conformational flexibility comparable to DNA (48-49). Apparently, both the eastern sugar pucker and conformational flexibility of the deoxyribonucleotide furanose ring favored by the enzyme, i.e., locking the sugar north or south resulted in slower cleavage rates.

Role of conformational flexibility within the catalytic site of the heteroduplex

Conformation flexibility was introduced at the catalytic site of the heteroduplex substrate with modifications exhibiting incrementally increasing flexibility with the hydrocarbon linkers predicted to exhibit the greatest degree of conformational flexibility followed by abasic and gancyclovair deoxyribonucleotides and the $\prod$ stacking deoxyribonucleotides, (e.g., 2-fluoro-6-methylbenimidazole, 4-methylbenimidazole and 2,4-difluorotolyl deoxyribonucleotides) (Fig. 1B). Table IB shows that with increased conformational flexibility at the catalytic site, the initial cleavage rates (V_0) and site-specific cleavage rates decreased. For example, the hydrocarbon linkers were predicted to exhibit the

greatest degree of conformational flexibility and were among the poorest substrates for RNase H1 activity. The site-specific rates for the ribonucleotide apposing these modification and the surrounding 3' and 5'-ribonucleotides were either significantly reduced or ablated resulting in initial cleavage rates (V_0) approximately two-fold slower than the unmodified substrate (Table I). The broad effect on the site-specific rates for the ribonucleotides surrounding the apposing ribonucleotide is likely due to the fact that the conformationally flexible linkers bridge both the 3' and 5'-deoxyribonucleotides. Interestingly, similar effects on the cleavage rates were observed for all three hydrocarbon linkers even through the linkers ranged in length from three to five carbons with the propyl and pentyl linkers predicted to be, respectively, shorter and longer than the length of the native deoxyribonucleotide linkage and the butyl linker predicted to most closely approximate the intraphosphate distance of the deoxyribonucleotide. The gancycloviar, abasic and tetrahydrofuran modified deoxyribonucleotides were also poor substrates for human RNase H1, although the site-specific cleavage rates for these heteroduplexes were slightly faster than the rates observed for the heteroduplexes containing the hydrocarbon linkers (Table IB). Taken together these data suggest that a conformationally rigid phosphate backbone is required for human RNase H1 activity. Furthermore, the slight improvement in the cleavage rates observed for the gancycloviar and abasic modifications compared with the hydrocarbon linkers suggests that the furansoe ring of the abasic deoxyribonucleotide and hydrogen bond base-pair formation of the gancycloviar modification likely offer modest conformational stability to the substrate.

In contrast, the ∏-stacking deoxyribonucleotides, (e.g., 2-fluoro-6-methylbenimidazole, 4-methylbenimidazole and 2,4-difluorotolyl deoxyribonucleotides) supported human RNase H1 activity (Table IB). Comparable initial cleavage rates and site-specific cleavage rates were observed for these heteroduplexes compared to the unmodified substrate. Interestingly, the site-specific cleavage rates for the second 3'-ribonucleotide were significantly slower suggesting that a stable hydrogen bond base-pair is required two base-pairs 5' to the scissile phosphate. The fact that the ∏-stacking deoxyribonucleotides exhibited comparable site-specific cleavage rates for the ribonucleotide apposing the modification compared with the unmodified substrate suggests that these modifications likely form favorable stacking interactions resulting in a stable helical conformation. In fact, previous studies have shown that the 2-fluoro-6-methylbenimidazole deoxyribonucleotide was an effective substitute of native deoxyribonucleotides. This modification was shown to efficiently incorporate into replicating DNA with DNA polymerase-→(50).

Interaction between enzyme and substrate at the catalytic site

The interactions between RNase H1 and the heteroduplex substrate at the catalytic site has been inferred by molecular modeling and site-directed mutagenesis of *E. coli* and human RNase H1 as well as the crystal structure of the *E. coli* enzyme (Fig. 3) (42). Specifically, site-directed mutagenesis suggests that the glutamine at position 72 of the *E. coli* enzyme forms a hydrogen bond with the 2'-hydroxyl of the ribonucleotide at position –1 and that the backbone imino and carbonyl groups of cysteine-13 were function as

proton donor and acceptor, respectively, in the hydrogen bonding interaction with the 2'-hydroxyl of the ribonucleotide at position +1 (42). The asparagine-16 and asparagine-44 residues of the enzyme were suggested to bind electrostatically with the phosphates of, respectively, the deoxyribonucleotides apposing the scissile ribonucleotide and the ribonucleotide at position –1. The aspartic acid residues at positions 10 and 70 were predicted to bind the 2'-hydroxyl of the scissile ribonucleotide via Mg^{2+} ion coordination (42). Finally, these amino acid residues are conserved in human RNase H1 and have been shown by site-directed mutagenesis to be required for activity (13, Lima unpublished).

The loss human RNase H1 activity observed for the heteroduplexes containing southern biased 2'-methylthiothymidine modification as well as the lack of cleavage observed for the northern biased 2'-alkoxy modified heteroduplexes is consistent with the predicted binding site for the enzyme (21-22, 27). Together, these data suggest that irrespective of sugar conformation, bulky 2'-substituents positioned in the minor groove of the heteroduplex substrate interfere with human RNase H1 cleavage. Similarly, the 2-thio substitution of 2-thiothymidine is predicted to be situated within the minor groove of the heteroduplex and the slower cleavage rates observed for these heteroduplexes may be the result of the sulfer either interfering with the enzyme sterically as a result of it's strong electronegative propreties.

The modified heteroduplexes examined here suggests that the width of the minor groove of the heteroduplex substrate is important for RNase H1 catalysis and that variations in minor groove width as a function of sugar pucker appear to obviate the proper positioning

of the enzyme on the heteroduplex substrate. For example, the heteroduplexes containing the 2'-ara-flouro deoxyribonucleotides, which produce a minor groove width comparable to the RNA/DNA heteroduplex, exhibited comparable human RNase H1 cleavage rates. On the other hand, deoxyribonucleotide modifications exhibiting a northern biased sugar conformation, (e.g., 2'-thiothymidine and cyclohexenylthymidine) reduced human RNase H1 activity. Consequently, the wider minor groove generated by these modifications likely precludes the associated metal ion coordination of the enzyme with the 2'-hydroxyl of the scissile ribonucleotide and electrostatic interaction with the phosphate of the modified deoxyribonucleotide (Fig. 3) (42). Similarly, a wider minor groove could account for the observed reduction in the site-specific rates for the adjacent 3'-ribonucleotide by preventing the formation of the putative hydrogen bond between the enzyme and the 2'-hydroxyl of the ribonucleotide at position +1 and the electrostatic interaction with the phosphate of the apposing deoxyribonucleotide (Fig.3). Consistent with these observations and the proposed model for the interaction of the enzyme with the heteroduplex substrate at the catalytic site, the significantly slower cleavage rates observed for the 4'-methylthymidine heteroduplexes suggest that proper positioning of the deoxyribonucleotide phosphate apposing the scissile ribonucleotide is critical for human RNase H1 cleavage. In addition, these observations suggest that the human RNase H1 activity associated with the deoxyribonucleotides exhibiting the northern verses southern biased sugar conformations is likely the result of differences in the relative positions of the inter- and intranucleotide phosphates on the heteroduplex substrate. Lastly, the cleavage rates observed for the 3'-methylthymidine modified heteroduplexes

suggest minor groove widths that are narrower than the RNA/DNA heteroduplex are tolerated better than are wider minor grooves by human RNase H1.

Conformational flexibility of the deoxyribose also appears to be an important structural feature of the heteroduplex substrate for human RNase H1 activity. The preferred eastern O4-*endo* sugar pucker observed for the DNA strand of the heteroduplex is the result of the nearly symmetrical potential energy barrier for both south and north sugar conformations exhibited by deoxyribonucleotides. Both the pseudouridine and 2'-arafluoro-deoxyribonucleotides exhibit conformational flexibility in the sugar and the heteroduplexes containing these modifications shwed cleavage rates comparable to the unmodified substrate. Furthermore, modifications exhibiting strong conformationally biased sugars, (e.g., 2'-fluoro-deoxyribonucleotides) were less efficiently cleaved by the enzyme.

Whereas conformational flexibility of the deoxyribose was preferred, flexibility in the in the phosphate backbone of the DNA strand inhibited human RNase H1 activity. Modifications such as the hydrocarbon linkers and abasic deoxyribonucleotides that permit free rotation of the phosphate moiety were shown to inhibited human RNase H1 activity. Again these data suggest that proper positioning the phosphate groups of the deoxyribonucleotide, presumably for electrostatic contact with the enzyme, is essential for human RNase H1 catalysis. The cleavage rates observed for the $\prod$ stacking deoxyribonucleotides suggest that stable base-stacking independent of hydrogen bond formation between the bases at the catalytic site appeared to offered sufficient rigidity to

the phosphate backbone. Taken together these data suggest that variation in sugar conformation is significantly better tolerated by human RNase H1 than conformational flexibility in the phosphate backbone.

Previous studies have shown that nucleotides exhibiting conformationally biased sugars, bias the sugar conformation of the surrounding deoxyribonucleotides. For example, the NMR structures of chimeric RNA-DNA/RNA heteroduplexes show that the deoxyribonucleotides adjacent to the RNA of the chimeric strand adopts the northern pucker of the RNA (51). The transmission the northern sugar conformation of the RNA into the adjacent deoxyribonucleotides is likely due the intrinsically flexible nature of the deoxyribose sugar. Furthermore, these data suggested that modifications resulting in higher conformationally biased sugar populations would have a greater influence on the structure of the surrounding deoxyribonucleotides and consequently a greater impact on human RNase H1 activity. The impact of the highly northern biased 2'-fluoro deoxyribonucleotide modification on the human RNase H1 activity of the surrounding ribonucleotides shown here, suggests that conformational transmission exhibits a modest influence on human RNase H1 activity compared to other factors such as steric bulk in the minor groove and conformational flexibility within the phosphate backbone. It is important to note that the heteroduplexes examined here contained single nucleotide substitutions with a conformationally biased sugar. We have observed that substituting contiguous stretches of modified nucleotides with conformationally biased sugars exhibits a greater influence on the human RNase H1 activity against adjacent deoxyribonucleotides (data not shown).

The structure of human RNase H1 shows that in addition to the catalytic domain shared with the *E. coli* homolog, human RNase H1 contains an RNA-binding domain at the N-terminus of the protein (9). Human RNase H1 appears to identify the first 3'-DNA/5'-RNA base pair to achieve the proper positioning of the catalytic domain slightly less than one helical turn from the RNA-binding domain (20). Only when the enzyme is bound at the correct site and the helical geometry appropriate, will the catalytic unit be positioned appropriately to cleave the RNA. As a result, altering the local helical geometry, (e.g., altering the minor groove width or the inter- and intranucleotide phosphate distances) at the catalytic site on the heteroduplex may have a global effect on the precise positioning of the catalytic region with respect to the RNA-binding domain of human RNase H1 required for catalysis. Because the enzyme is predicted to position the catalytic domain 3' on the RNA relative to the RNA-binding domain, consistent with the results presented here, a local change in duplex geometry at the catalytic site on the substrate would impair the human RNase H1 activity at the adjacent 3'-ribonucleotides to the modification.

Implications for the design of antisense oligonucleotides

The demonstration that human RNase H1 plays a dominant role in the activities of DNA-like ASOs suggests that additional studies that explore the substrate preferences, enzymology, and regulatory processes for RNase H1 should support improved design of antisense agents. The demonstration that increases in RNase H1 activity correlated with increases in potency suggests that recruitment of RNase H1 to the ASO-RNA duplex

and/or cleavage of the RNA by the enzyme is limiting for ASO activity. Any strategy that would improve these processes should improve ASO potency (Wu, in press).

With respect to ASO design, the results presented here suggest that the preferred properties for the modified oligodeoxyribonucleotide include: 1) a conformationally flexible sugar producing an O4-endo pucker when hybridized to RNA; 2) no steric 2'-substituents; and 3) a conformationally rigid phosphate backbone. Clearly, the 2'-ara-fluoro, pseudouridine, 3'-methyl and ∏-stacking modified deoxyribonucleotides exhibit many of these qualities. Whether homogenously modified oligonucleotides containing these modifications support human RNase H1 remains to be determined. In the case of the 2'-ara-fluoro modification, fully modified ASOs were shown to be significantly less potent than phosphorothioate-linked DNA counterparts in reducing the cellular target levels (52). In light of the fact that none of the modifications tested were shown to enhance human RNase H1 activity compared with native deoxyribonucleotides and that these modification offer no clear advantage over native deoxyribonucleotides with respect to either duplex stability or nuclease resistance, other strategies to improve the potency ASO should be considered. For example, the calculated placement of these modifications in chimeric ASOs may be an effective means to improve human RNase H1 activity by potentially blocking the conformational transmission of 2'-alkoxy deoxyribonucleotide into the deoxyribonucleotide region of the chimeric ASO.

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Figure legends

Figure 1: **Position and structure of the deoxyribonucleotide and intranucleotide linker modifications** (A) Modified nucleotides containing conformationally biased sugar puckers. The northern biased modifications include: 2-thiothymidine (S^2T), cyclohexenylthymidine (CeNA) and 2'-fluorothymidine (2'-F). The southern biased modifications include: 2'-methylthiothymidine (2'-S-methyl), 4'-methylthymidine (4'-methyl), 3'-methylthymidine (3'-methyl), and pseudouridine. 2'-ara-fluoropyrimidine (2'-ara-Fluoro) represents the eastern biased sugar modification. (B) Structures of the modifications designed to introduce conformational flexibility into the heteroduplex. These modifications include: the propyl (C_3), butyl (C_4) and pentyl (C_5) hydrocarbon linkers; tetrahydrofuran (THF), abasic and gancyclovair (G_v) modifications; and the Π stacking 2-fluoro-6-methylbenimidazole (2-F-6-Me-ben), 4-methylbenimidazole (4-Me-ben) and 2,4-difluorotolyl (2,4-F-tolyl) deoxyribonucleotides. (C) The (x) shows the

position of the modification for the respective oligodeoxyribonucleotide. The positions are numbered 5'→3' on the oligodeoxyribonucleotide.

Figure 2: **Human RNase H1 cleavage pattern and site-specific rates for modified heteroduplex substrates.** (A) Polyacrylamide gel analysis of the unmodified substrate and heteroduplexes containing the 4'-methylthymidine (4'-methyl) modification at position T_9 and the 2'-thiothymidine (S^2T) at position T_8. The substrates were incubated in the absence (Lanes 1, 7 and 13) presence of human RNase H1 for: 5 min. (Lanes 2, 8 and 14); 10 min (Lanes 3, 9 and 15); 15 min (Lanes 4, 10 and 16); 30 min. (Lanes 5, 11 and 17); and 60 min. (Lanes 6, 12 and 18). The ribonucleotide positions are numbered accordingly: the ribonucleotide apposing the modification (0); the first (-1) and second (-2) ribonucleotides 5' to the modification; and the first (+1) and second (+2) ribonucleotides 3' to the modification. (B) Site-specific cleavage rates for unmodified substrate (gray bar), the 4'-methylthymidine modification at position T_9 (black bar) and the 2'-thiothymidine at position T_8 (hashed bar). Initial rates for each site on the heteroduplex were determined as described for multiple-turnover kinetics in Material and

Methods. The V_0 values are an average of three measurements with estimated errors of CV < 10%.

Figure 3: **Model for the interaction of RNase H1 with the heteroduplex substrate at the catalytic site.** The designations for ribonucleotide positions are described in Figure 2. The positions of the amino acid shown are for *E. coli* RNase H1 and the positions of the conserved amino acid are in the human enzyme are shown in brackets.

Table I: **Relative initial cleavage rates and site-specific for the modified heteroduplex substrates.** The position and description of the modifications are shown in Fig. 1. Initial and site-specific cleavage rates were determined as described in the Materials and Methods. [1] Ratio site-specific cleavage rates represents the initial cleavage rates for the modified heteroduplexes divided by the unmodified substrate. [2] Ratio V_0 represents the initial cleavage rates for the modified heteroduplexes divided by the unmodified substrate. [3] The designations for ribonucleotide positions are described in Figure 2. [4] Dashed lines indicate positions not cleaved by human RNase H1 for the unmodified

substrate and modified heteroduplexes, i.e., terminal five 5'-ribonucleotides and five 3-ribonucleotides (see Fig. 2B). The V_0 values are an average of three measurements with estimated errors of CV < 10%.

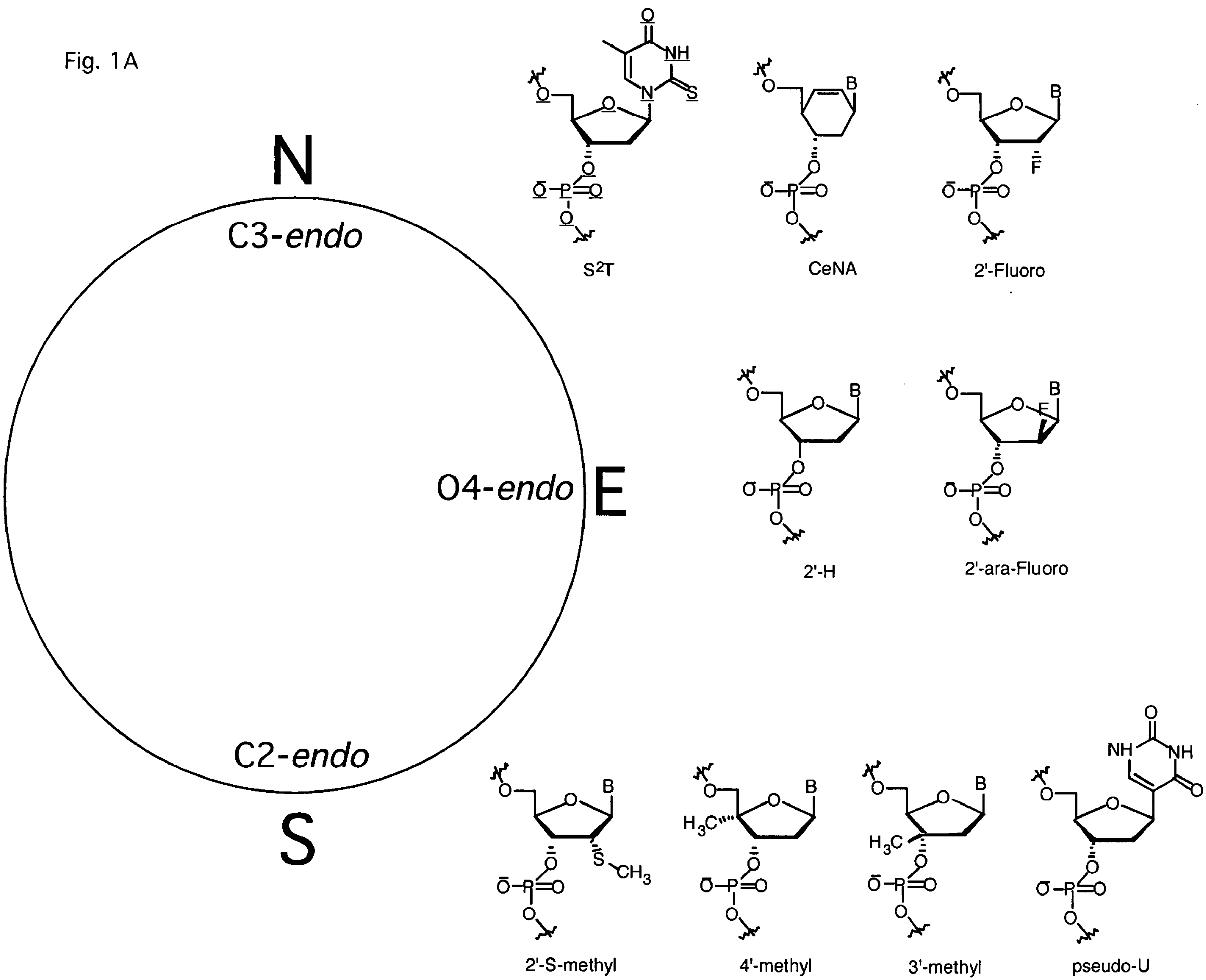
Table IA

Modification	Position of modification	Ratio Site-Specific Cleavage Rate[1] (modified/ unmodified)					Ratio V_0[2] (modified/unmodified)
		-2	-1	0[3]	+1	+2	
2'-ara-Fluoro	T_7	0.9	1.1	0.9	1.0	--[4]	1.1
	T_8	1.3	1.5	0.8	1.5	1.50	1.1
	T_9	1.0	1.1	1.5	0.6	0.4	1.1
	C_{10}	0.7	1.1	0.8	1.0	0.6	1.2
	C_{13}	1.1	0.7	0.6	0.8	0.6	1.1
	C_{15}	--[4]	--[4]	1.1	1.3	0.4	1.1
Pseudouridine	T_7	0.8	0.7	0.6	0.6	--[4]	0.9
	T_8	1.4	1.3	0.7	0.2	0.3	1.1
	T_9	1.0	0.8	1.0	0.2	0.2	1.2
2'-Fluoro	T_7	0.5	0.2	0.2	0.1	--[4]	0.6
	T_8	0.8	0.6	0.2	0.3	0.6	0.7
	T_9	1.1	0.9	0.2	0.3	0.6	0.7
S^2T	T_7	0.4	0.4	0.2	0.1	--[4]	0.5
	T_8	1.1	0.6	0.0	0.0	0.7	0.7
	T_9	1.3	1.0	0.3	0.3	0.9	0.7
2'-S-Methyl	T_7	0.6	0.6	0.1	0	--[4]	0.5
	T_8	0.5	0.6	0.1	0	0	0.4
	T_9	0.7	0.6	0.2	0.1	0.3	0.4
4'-Methyl	T_7	0.5	0.3	0	0	--[4]	0.5
	T_8	0.3	0.1	0	0	0	0.6
	T_9	0	0	0	0	0	0.3
3'-Methyl	T_7	0.3	0.7	1.0	0.8	--[4]	0.8
	T_8	1.6	0.8	0.7	0.1	0.1	0.8
	T_9	1.3	0.7	0.6	0.2	0	0.7
CeNa	T_7	0.6	0.8	0.8	0	--[4]	0.4
	T_8	0.7	0.9	0.6	0	0	0.4
	T_9	0.3	1.0	0.5	0	0	0.4

Table IB

Modification	Position of modification	Ratio Site-Specific Cleavage Rate[1] (modified/ unmodified)					Ratio V_0[2] (modified/unmodified)
		-2	-1	0[3]	+1	+2	
Propyl linker	A_{12}	0.6	0	0	0	0	0.5
	G_{14}	--[4]	0	0	0	0	0.6
	C_{15}	--[4]	--[4]	0	0	0	0.5
Butyl linker	A_{12}	0.5	0.1	0	0	0	0.5
	C_{15}	--[4]	--[4]	0.1	0	0.6	0.5
Pentyl linker	A_{12}	0.1	0.1	0	0.5	0.9	0.6
	C_{15}	--[4]	--[4]	0.1	0	0.9	0.5
Tetrahyrofuran	A_{12}	0	0	0	0.6	0.5	0.4
	G_{14}	--[4]	0.5	0	0.1	0	0.4
Abasic	T_9	0.6	0.3	0.2	0.3	0.2	0.6
	C_{10}	0.5	0.4	0.5	0.3	0.1	0.6
	C_{11}	0.6	0.3	0.2	0.3	0.2	0.5
Gancyclovir	G_{14}	--[4]	0.1	0.5	0.3	0.4	0.4
4-F-6-Me-ben	G_{14}	--[4]	0.8	0.8	0.9	0.9	0.8
4-Me-ben	A_{12}	0.7	0.7	0.9	0.9	0.5	0.8
2,4-F-tolyl	T_9	0.9	1.0	1.3	0.8	0.2	0.8
	C_{10}	1.3	1.5	1.0	1.0	0.1	0.9
	C_{11}	1.3	1.1	0.8	0.7	0.5	1.1

Fig. 1A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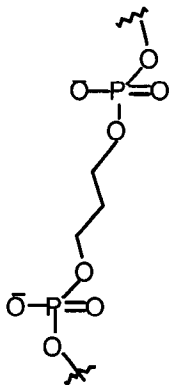
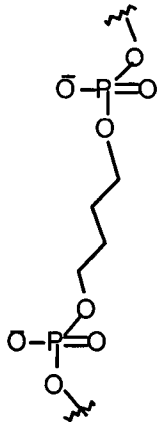
C3-endo
O4-endo
E
C2-endo
S
S2T
CeNA
2'-Fluoro
2'-H
2'-ara-Fluoro
2'-S-methyl
4'-methyl
3'-methyl
pseudo-U

Fig. 1B



C_3



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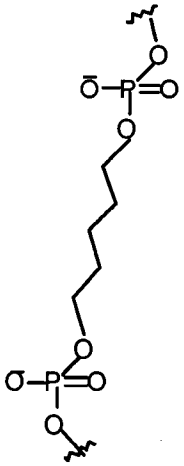

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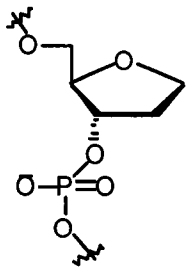

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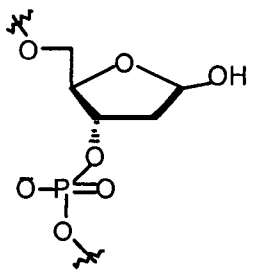
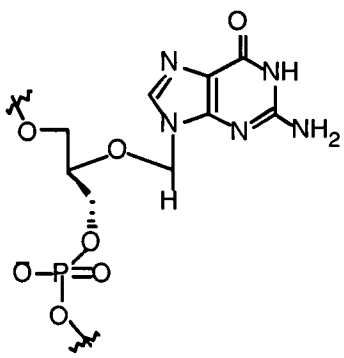
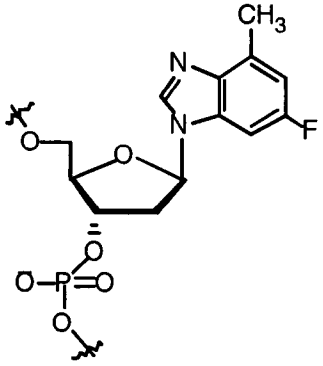


abasic



GV



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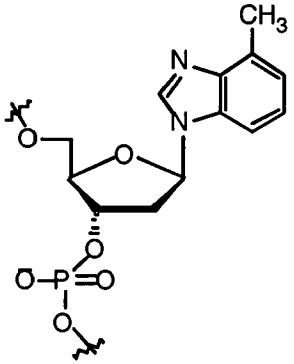
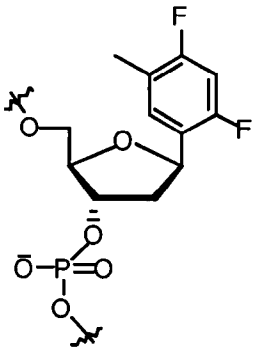


4-Me-ben



2,4-F-tolyl

Fig. 1C

T_7 : CTACGCxTTCCACGCACAGT

T_8 : CTACGCTxTCCACGCACAGT

T_9 : CTACGCTTxCCACGCACAGT

C_{10}: CTACGCTTTxCACGCACAGT

C_{11}: CTACGCTTTCxACGCACAGT

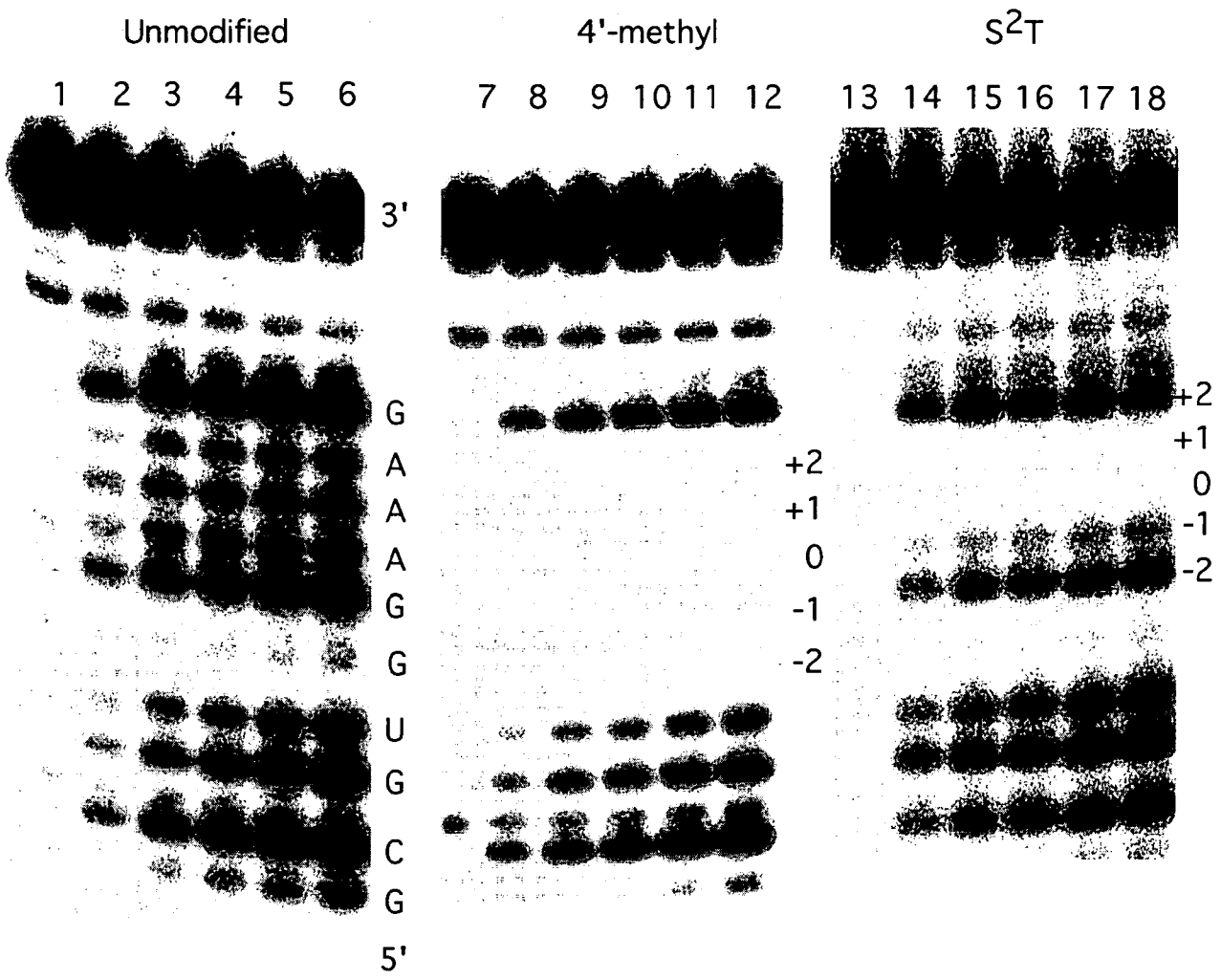
A_{12}: CTACGCTTTCCxCGCACAGT

C_{13}: CTACGCTTTCCAxGCACAGT

G_{14}: CTACGCTTTCCACxCACAGT

C_{15}: CTACGCTTTCCACGxACAGT

Fig. 2A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13 14 15 16 17 18
3'
G
A
A
A
G
G
U
G
C
G
5'
+2
+1
0
-1
-2
+2
+1
0
-1
-2

Figure 2B

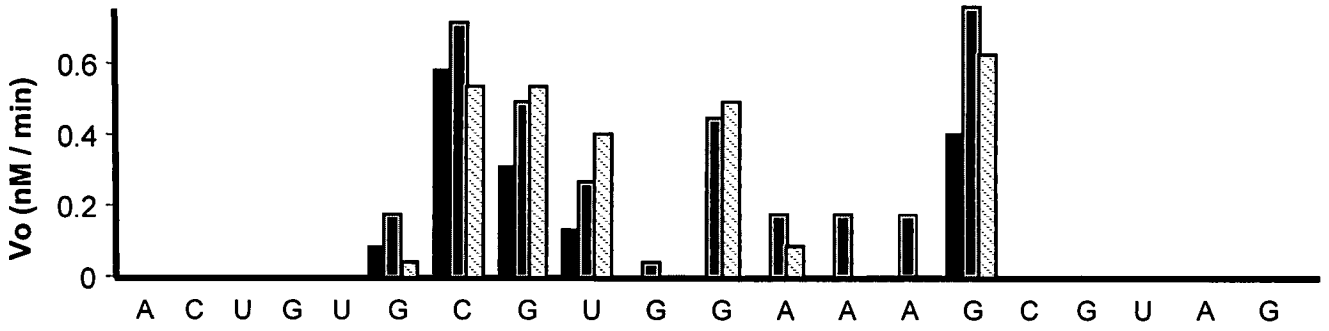
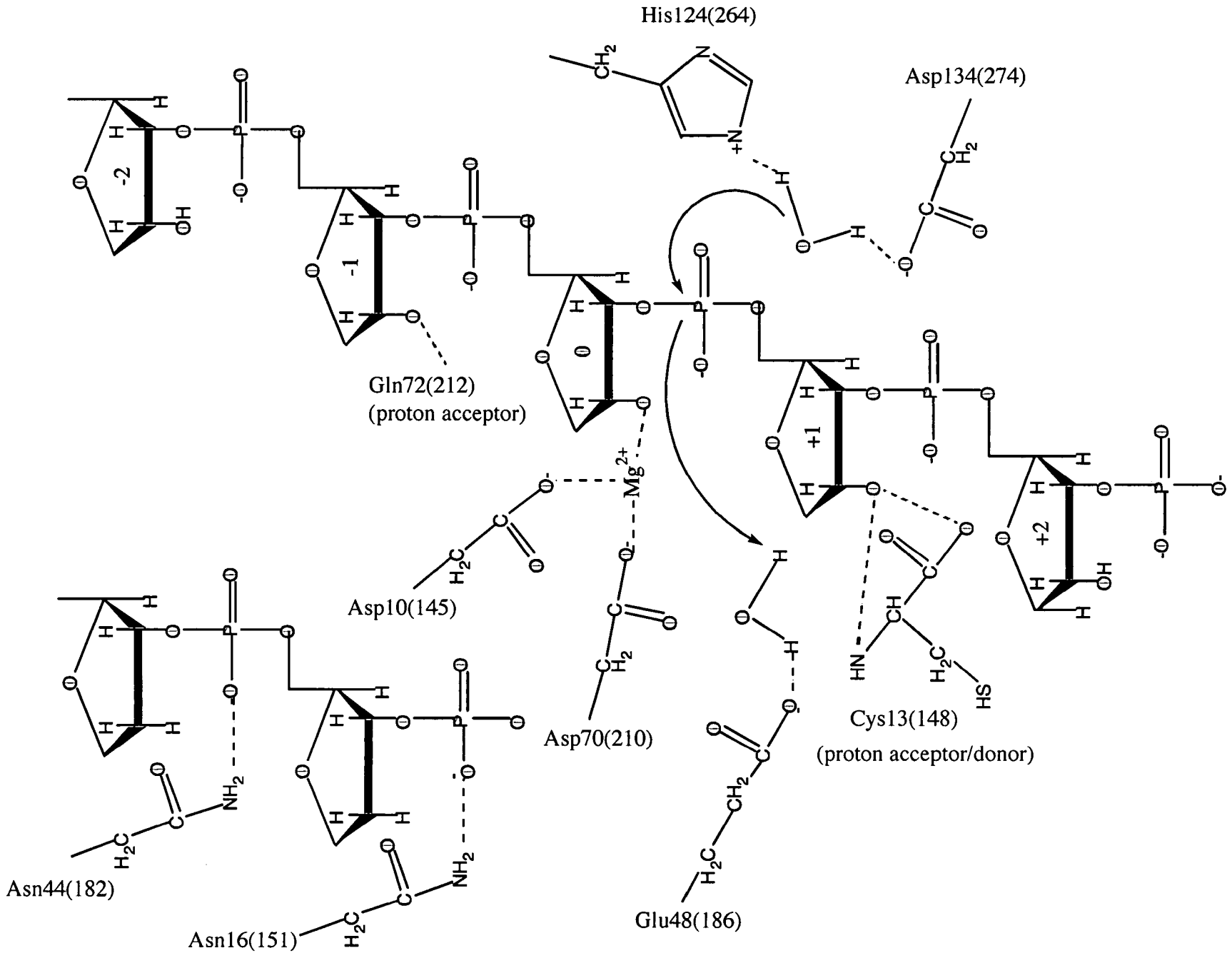


Fig. 3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